Adhesion Forces between LewisX Determinant Antigens as Measured by Atomic Force Microscopy**

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A carbohydrate coat named glycocalix covers the outer membrane of most types of cells. These carbohydrates at the cell surface are responsible for communication with other cells and with the extracellular matrix through interaction with proteins.[1] Recently, carbohydrate to carbohydrate interactions have been proposed as the initial step of cell adhesion.[2-3] Embryogenesis, metastasis, and other proliferation processes are, according to Hakomori,[4] mediated by carbohydrate – carbohydrate interactions. Some of the structures involved in this novel mechanism are the trisaccharide LewisX determinant (Galβ1→4[Fucα1→3]GlcNAcβ1), and the glycosphingolipids GM3 (NeuAcα2→3Galβ1→4Glcβ1Cer) and Gg3 (GalNAcβ1→4Galβ1→4Glcβ1Cer). The LewisX antigen (LeX)—previously defined as the stage-specific embryonic antigen SSEA-1—is found in a wide variety of systems such as human cancers, pre-implantation mouse embryos, embryonic carcinoma cells, and human erythrocytes. This antigen mediates, by a homotypic interaction, morula compaction and teratocarcinoma metastasis in mouse cells.[2-5] A heterotypic interaction between GM3 and Gg3 also seems to be involved in metastasis of melanoma cells in mice.[6-7] Characteristic features of these interactions are a strong dependency on divalent cations (Ca2+), a high specificity and a low affinity. Nature solves these problems by a multivalent presentation of the carbohydrate antigen at the cell surface. These characteristics make it difficult to study and to quantify this process.[8,9] Nevertheless, in previous studies using synthetic receptors we have shown that the interaction between lipophilic patches of simple carbohydrate units in two dimensional (2D), self-assembled monolayer (SAM) surfaces, the study demonstrates the self-recognition ability and selectivity of the LeX antigen. To measure these adhesion forces, flat gold surfaces were functionalized with the LeX determinant containing neoglycoconjugate 2 to form self-assembled monolayers. For comparison purposes a parallel study was carried out by similarly functionalizing gold surfaces with lactose (Galβ1→4Glc) containing neoglycoconjugate 1 (Figure 1). Neoglycoconjugates 1 and 2 were synthesized (manuscript in preparation) by glycosidation of conveniently protected lactose and LeX derivatives, respectively, with 11- mercapto undecanol using the trichloroacetimidate method.[20] The thiol group was incorporated to attach these molecules to the gold surfaces. A micro-fabricated AFM cantilever[21] and a gold-surface sample were functionalized with neoglycoconjugates 1 and 2, and schematic representation of the sample and the AFM tip which is functionalized with self-assembled monolayers of the neoglycoconjugates.

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in 1 mM solutions of 1 and 2 in methanol and water, respectively, then rinsed several times with the same solvent and dried under a stream of nitrogen.

The neoglycoconjugates organize themselves as monolayers on both the tip and on the sample. Although this model may be considered far away from the natural glycosphingolipids presentation, it provides a polyvalent functionalized surface with chemically well-defined structure to evaluate carbohydrate–carbohydrate interactions. In a typical procedure, a series of approach and retract cycles with the Le<sup>S</sup> functionalized tip and sample were carried out in water and in 10 mM aqueous calcium solution in a liquid cell. Unloading rates of about 100 nm s<sup>-1</sup> were used. For each force scan, the functionalized tip was maintained in contact with the molecular surface at a constant force (150 pN) for two seconds before unloading to ensure intermolecular contact and chemical equilibrium. The unloading curve, representing the adhesion force as a function of the distance, shows a step-like behavior (Figure 2). Each step corresponds to an out of equilibrium cantilever movement because of the unbinding events among the molecules of the tip and the molecules of the sample. Measurement of the step height leads to the determination of the intermolecular unbinding force. For each sample, more than 300 curves over 15 different areas of the sample surface were recorded.

Typical force–distance curves obtained with a tip and a sample functionalized with Le<sup>S</sup> determinant are presented in Figure 2A and 2D. The stepwise profile of the curves is characteristic of molecular interaction, and was observed in water as well as in calcium solution (Figure 2A and 2D, respectively). A statistical analysis of the step height reveals that the measured unbinding forces are entire multiples of 20 ± 4 pN (Figure 2B and 2E). The strong periodicity of the auto-correlation function (Figure 2C and 2F) is an important evidence that the interaction observed in these experiments results exclusively from specific adhesion forces. The 20 ± 4 pN force has been assigned to the binding force between two individual Le<sup>S</sup>-determinant molecules. The value of the force was independent of the presence of calcium ions in the solution. This result was not expected based on the described characteristics of carbohydrate–carbohydrate interactions. However, in experiments using many different tips and different samples the same 20 ± 4 pN interaction force was obtained.

Carbohydrate to carbohydrate interaction is based mainly on van der Waals contacts between the corresponding polyamphiphilic surfaces involved in the process. The complementary nature of these surfaces makes the interaction highly specific and calcium ions seem to be crucial for this process to occur in biological and other model systems. The role of calcium in carbohydrate–carbohydrate interactions is not well understood. Calcium ions could be responsible for the approach and organization of the sugar moieties which provide the adequate surfaces for interaction, or may enhance the adhesion force between complementary surfaces, acting as a bridge between specific hydroxy groups.

In the AFM experiments presented here, the formation of a well-packed self-assembled monolayer provides the organization of carbohydrates on the surfaces. Furthermore, interaction between the molecules is established by applying an initial contact force in the cantilever which makes the Le<sup>S</sup>-functionalized surfaces approach. Therefore, if the role of calcium ions is to favor the approach and organization of the molecules at the surface, it could not be detected in the AFM experiment. However, if the role of calcium is to reinforce the contact between the interacting molecules, an increase in the

Figure 2. A) and D) Typical AFM force–distance curves obtained between an Le<sup>S</sup>-functionalized tip and a SAM of Le<sup>S</sup> molecules in water and in 10 mM calcium solution, respectively, B) and E) histogram of the unbinding force measured through 300 force–distance curves of Le<sup>S</sup> molecules in water and in 10 mM calcium solution, respectively, and C) and F) corresponding auto-correlation function.
occurs in the presence of calcium cations. In other words, the interaction between the LeX molecules or vice-versa gave the same force–distance curves as in water or in aqueous calcium solution (Figure 3:B). No interaction was observed either in water or in aqueous calcium solution. This value is the smallest adhesion force reported to date. However, it is in the same order of magnitude as the forces of a single binding event measured in the proteoglycan of marine sponges[26] and other systems such as cadherine–cadherine,[16] adenosine–thymine,[25] glycosphingolipid GM1–cholera toxin B (CTB)[26] and the ferritin–antiferritin antigen–antibody system.[17] A further step to obtaining thermodynamic information would be to correlate the measured forces with the energy of binding. Moy et al.[15] and Chilkoti et al.[27] found a linear correlation between experimental rupture forces and enthalpy in different avidin and biotin derivatives. Preliminary studies based on weak affinity chromatography have allowed us to measure a LeX–LeX binding constant around 10 M⁻¹ at room temperature (unpublished results). This value corresponds to an energy of 5.7 kJ mol⁻¹ and according to the equation \( E = Fd \), the interaction distance could be determined taking into account that the maximum force \( (F) \) found by AFM is 20 pN.[28] The distance \( (d) \) obtained, using this approach,[28] is 0.5 nm which approximately corresponds to the size of a pyranose ring of the LeX trisaccharide.

In summary, we have been able to quantify the adhesion forces between polyvalent surfaces of LeX trisaccharide by using atomic force microscopy. The adhesion force measured between two LeX molecules (20 ± 4 pN) represents a new limit in the sensitivity of force microscopy. The results obtained establish the capacity and selectivity of LeX antigen for self-recognition supporting the concept of carbohydrate–carbohydrate interactions as a mechanism for cell adhesion and recognition. Furthermore, these results confirm AFM as a powerful tool to measure weak adhesion forces. The measurements of adhesion forces using other carbohydrate antigens are in progress.

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The CH$_3$N Diradical: Experimental and Theoretical Determinations of the Ionization Energies**

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Methylnitrene, the CH$_3$N diradical, is one of the most interesting of all organic free radicals, and it has drawn a lot of attention in both experiments and theory. As the simplest alkyl nitrene, the general class of molecules with the form RN, CH$_3$N is a dielectric radical similar to carbene CH$_2$: and is thought to be an important intermediate in many organic and inorganic reactions. Berry suggests that the alkyl nitrene should have electron states similar to NH which has been well characterized by spectroscopists. However, much less is known about alkyl nitrenes such as CH$_3$N, CH$_2$CH$_2$N, (CH$_3$)$_2$CH–N, or (CH$_3$)$_3$C–N. There are no reports on the ionization energy of the nitrenes until now, but these ionization energies, especially the first ionization energy, are important physical and chemical data.

When combined with ab initio molecular orbital calculations, HeI photoelectron spectroscopy (PES) gives a lot of information on the electronic and vibrational structures, the configuration, the bonding characteristics, as well as the relative photoionization cross sections of different molecular orbitals. Of course, the most important information obtained from the PES study is the ionization energy of the different molecular orbitals. Pople and their co-workers pointed out that the ab initio Gaussian 2 (G2) calculation can correctly predict the first ionization energy.

We have shown how to generate a continuous flowing beam of short-lived species by the microwave discharge or pyrolysis of the parent species, allowing us to perform PES studies on reactive open-shell species such as NO$_2$, (CH$_3$)$_2$N[14] CH$_2$O[19] CH$_2$S[18] CH$_2$SS[20] CH$_2$(CH$_2$)$_2$S[21] (CH$_3$)$_2$CO[22] and further demonstrated that the G2 calculation can also predict the ionization energies of different ionic states. Herein, we present the experimental and theoretical determination of the ionization energies of different ionic states for the CH$_3$N diradical; this is also the first report on ionization energy for the nitrenes.

Calculations on the CH$_3$N$_2$ molecule indicate that the bond between the CH$_3$N and N$_2$ units is the weakest present and therefore pyrolysis of the CH$_3$N$_2$ offers a simple route to the CH$_3$N diradical through loss of the N$_2$ unit. However, in the pyrolysis study of CH$_3$N$_2$ carried out by Bock and co-workers,[23–26] no PES signals of the CH$_3$N$_2$ diradical could be detected and PES signals attributed to CH$_2$NH appeared when CH$_3$N$_2$ was heated to 770 K.

Through careful analysis of the experimental process used by Bock et al.[23–26] it is clear that: 1) the distance from the point of pyrolysis of the parent compound to the photo-ionized point for the new species generated is too far (maybe more than half meter); 2) the pyrolysis temperature (770 K) for maintaining the new species is also too high. Obviously, it is difficult to record the PES signals of transient species which have a short life-time, such as the CH$_3$N diradical. In fact, mixed PE spectra showing CH$_3$N$_2$ and CH$_2$NH (these spectra are the same as Figure 1 reported by Bock and Ralph in ref. [26]) were always obtained from the pyrolysis of the CH$_3$N$_2$ at 650$^\circ$C with the heater installed outside our PE spectrometer. When the PES of the species generated by pyrolysis of CH$_3$N$_2$ are recorded in situ (see Experimental Section) a lot of the short-lived active species is detected at a lower temperature in our PE spectrometer.

Figure 1 gives the PE spectrum of the product obtained from the pyrolysis of CH$_3$N$_2$ at 145 $^\circ$C, the spectrum is recorded in situ under protection of the NO species. Note-worthy is that the mixed PE spectra showing both CH$_3$N$_2$ and CH$_2$NH are also obtained at 215 $^\circ$C if NO is not.


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